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Comparative evaluation of six ELISAs for the detection of antibodies to the non-structural proteins of foot-and-mouth disease virus

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Abstract

To validate the use of serology in substantiating freedom from infection after foot-and-mouth disease (FMD) outbreaks have been controlled by measures that include vaccination, 3551 sera were tested with six assays that detect antibodies to the non-structural proteins of FMD virus. The sera came from naïve, vaccinated, infected and vaccinated-and-infected animals; two-thirds from cattle, the remainder from sheep and pigs. The assays were covariant for sensitivity, but not necessarily for specificity. A commercial kit from Cedi-diagnostics and an in-house assay from IZS-Brescia were comparable to the NCPanaftosa-screening index method described in the Diagnostic Manual of the World Animal Health Organisation. Using these three tests the specificity and sensitivity for the detection of carriers in vaccinated cattle approaches or exceeds 99% and 90%, respectively.

Keywords: Foot-and-mouth disease; Non-structural proteins ELISAs; Tests performances

1. Introduction

The European Council directive 2003/85/EC, which prescribes measures for intra-community control of foot-and-mouth disease (FMD), makes provision for a "vaccinate-to-live" policy in the event of a future FMD outbreak in an EU member state. However, diagnostic tests, which could be used in support of this policy, must be validated for this purpose. An ELISA that detects antibodies to non-structural proteins of the FMD virus (NSP-ELISA) could be used to discriminate between infected and non-infected animals regardless of their vaccination status, and thereby help countries to substantiate absence of infection. This could enable countries to regain their previous FMD-free status after emergency use of FMD vaccine, without the need to slaughter all vaccinated animals; hence "vaccinate-to-live". The World Animal Health Organisation (Office International des Epizooties, OIE) has

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adopted an NSP-ELISA developed by PANAFTOSA, as its index screening method for discrimination purposes [1]. This test, complemented by a confirmatory assay, is used in South America to demonstrate FMD freedom post-vaccination [2]. At present, in Europe, there are four commercially available NSP-ELISAs and other "in-house" tests. Although each of these NSP-ELISAs has been evaluated at different times and in different laboratories using different sera, none had been sufficiently validated for use in support of a vaccinate-to-live policy.

Validation requires large panels of sera representative of different livestock species that have been vaccinated or vaccinated-and-infected with different serotypes of FMD virus. Therefore, an EU-funded research group, a consortium of European FMD reference laboratories and PANAFTOSA, have pooled their resources to compare the performance of the NSP-ELISAs currently available in Europe with one another and with that of the OIE index test when used to test sera from cattle, sheep and pigs. A total of 3551 sera from Belgium, Denmark, Germany, Israel, Italy, The Netherlands, Turkey, the UK, South America and Zimbabwe were assembled for testing at a workshop in Brescia, Italy during May 2004, where all of the sera were tested in parallel by each of the six ELISAs. Bovine sera constituted a large proportion of the total sera tested during this workshop (2579 sera or 67% of the total number tested) such that sufficient data is now available to complete the comparative evaluation of these tests for use with cattle. However, more sera for testing will have to be sourced from sheep and pigs before the process can be completed for these species.

The present paper presents analyses of the data derived from the bovine sera tested during this workshop. In addition to estimation of the diagnostic sensitivities and specificities for each test method with different serum categories, correlation and discrepancy analyses are presented along with an evaluation of analytical sensitivities using serial dilutions of reference sera derived from infected cattle. Results are also presented for preliminary analyses of the sheep and pig data.

2. Materials and methods

2.1. Tests

Six different NSP-ELISAs were compared, these were: (1) NCPanaftosa-screening from PANAFTOSA [3]; (2) 3ABC trapping-ELISA from IZS-Brescia [4]; (3) Ceditest® FMDV-NS (Cedi Diagnostics B.V., Lelystad, The Netherlands [5,6]); (4) SVANOVIR™ FMDV 3ABC-Ab ELISA (Svanova, Upsala, Sweden [7]); (5) CHEKIT-FMD-3ABC (Bommeli Diagnostics, Bern, Switzerland [8,9]); (6) UBI® FMDV NS ELISA (United Biomedical Inc., New York, USA [10]). Five tests detect antibodies to the viral non-structural polypeptide 3ABC, expressed as recombinant antigen in different expression systems, while the UBI kit recognises antibody to a 3B synthetic peptide. Methodologically, the Ceditest is a blocking ELISA and can be applied to any animal species; the other five tests are indirect ELISAs and use anti-species conjugates. In four tests, purified antigens are directly coated to microplates, while in the IZS-Brescia and Ceditest ELISA the 3ABC antigens are trapped by a different monoclonal antibody. The ELISA from PANAFTOSA is the OIE Index test and is described in the OIE 'Manual of standards for diagnostic tests and vaccines' [1]; the description of the commercial ELISAs can be found in the test manuals on the website of the respective companies (http://www.cedi-diagnostics.com; http://www.bommeli.com; http://www.unitedbiomedical.com; http://www.svanova.com). The kit from Svanova was still a prototype at the time the comparison was performed. The ELISA from IZS-Brescia is an in-house test, performed as previously described [4] with the following modifications: test sera were diluted 1/100, the conjugate for ruminants was a mixture of two

Table 1
NSP antibody detection ELISAs evaluated at the workshop

Test-kit	Manufacturer/supplier	Format	Antigen/expression system	Species	Conjugates	Threshold[a]
NCPanaftosa-screening	Panaftosa, PAHO, Rio de Janeiro, Brazil	Indirect ELISA (antigen coated)	3ABC/*E. coli* (MS2 fusion protein)	Bovine, ovine, swine	Anti-bovine, anti-ovine, anti-swine	≥10 PP[b]
3ABC trapping-ELISA	IZSLER, Brescia, Italy	Indirect ELISA (antigen-capture)	3ABC/*E. coli* (MS2 fusion protein)	Bovine, ovine, swine	Anti-ruminants, anti-swine	≥10 PP
Ceditest® FMDV-NS	Cedi diagnostics B.V., Lelystad, The Netherlands	Blocking ELISA (antigen-capture)	3ABC/Baculovirus	All species	Anti-3B monoclonal antibody	≥50 PI[c]
SVANOVIR™ FMDV 3ABC-Ab ELISA	Svanova, Upsala, Sweeden	Indirect ELISA (antigen coated)	3ABC/*E. coli*	Bovine	Anti-bovine	≥48 PP
CHEKIT-FMD-3ABC	Bommeli Diagn/Idexx, Bern, Switzerland	Indirect ELISA (antigen coated)	3ABC/*E. coli* (GST fusion protein)	Bovine, ovine, swine	Anti-ruminants, anti-swine	≥20 PP
UBI® FMDV NS ELISA	United Biomedical Inc., New York, USA	Indirect ELISA (antigen coated)	3B synthetic peptide	Bovine, swine	Protein A/G, anti-swine	≥23 PP

[a] For ELISAs that allow for a "grey zone" (NCPanaftosa-screening, CHEKIT-FMD-3ABC) the lower cut-off was used as threshold of positivity.
[b] PP: percent positivity, compared to positive control.
[c] PI: percent inhibition, compared to negative control.

Table 2
The origin of cattle sera tested by all six ELISAs, according to their infectious, vaccination and carrier status

Origin	Non-infected		Infected				Post-outbreak	Total
	NV	V	NV		V			
			C+	C−/?	C+	C−/?		
Belgium	96	48	–	–	–	–	–	144
Denmark	100	–	–	–	–	–	–	100
Germany	–	191	–	–	36	42	–	269
Italy	192	46	–	15	–	72	–	325
The Netherlands	22	74	6	6	73	55	–	236
United Kingdom	263	63	15	20	99	156	–	616
South America	2	3	–	–	17[F]		–	22
Israel	–	–	–	–	–	–	465	465
Zimbabwe	–	–	–	–	–	–	402	402
Total sera	675[F]	425	21	41	225	325	867[F]	2579
Total cattle	672	425	17	37	67	218	867	2303

Sera originated from experimental cattle, except those super-marked with an F that originated from field. NV, non-vaccinated; V, vaccinated; C+, carrier; C−/?, carrier negative or carrier undefined; F, field origin.

monoclonal antibodies, one reactive with ruminant IgG1, the other specific for bovine IgG2, for pigs a polyclonal anti-pig immunoglobulin conjugate was used, the results were expressed as percentages of an internal positive control.

Summarizing information on each of the ELISAs is provided in Table 1.

Samples were tested singly (i.e. applied to one well of an ELISA plate, or pair of wells in case of use of a negative antigen well) during the workshop held at the FMD laboratory of the IZSLER in Brescia (Italy), by a unique technical group composed by personnel from five National laboratories. Spectrophotometric reading of optical densities and expression of results according mathematical formula (as indicated in each test manual) excluded any possibility of subjective interpretation. Sera that gave results discordant in one or more assays were later re-tested, but unless specified otherwise, the analyses presented were derived from the results of the first testing, performed simultaneously with the six ELISAs. For those ELISAs that allow for a "grey zone" of interpretation comprising doubtful results, the lower threshold was used for expression of results (i.e. reactions equal or above the lower threshold were scored as positive).

2.2. Samples

A total of 2579 cattle sera, 703 sheep sera and 269 pig sera were examined. The country of origin and the categories with respect to vaccination and infection status are indicated in Tables 2–4.

2.2.1. Cattle sera

The cattle sera originated from eight countries, Belgium, Denmark, Germany, Israel, Italy, The Netherlands, the United Kingdom (UK) and Zimbabwe and from South America (Table 2). The sera came from FMDV-naïve cattle or from cattle vaccinated and/or exposed to one of the following six serotypes of FMDV: O, A, Asia 1, C, SAT 1, SAT 2. The

Table 3
Time post-infection intervals of cattle sera

Days post-infection class	Infected				Total
	Non-vaccinated		Vaccinated		
	C+	C−/?	C+	C−/?	
7–14	1	4	31	150	186
15–27	13	16	50	96	175
28–100	6	20	90	63	179
>100	1	1	54	16	72
Total	21	41	225	325	612

C+, carrier (a serum sample was attributed to the carrier category when originated from a cattle that was proven to have developed a persistent infection); C−/?, carrier negative or carrier undefined.

Table 4
Sheep and pig sera

	Non-exposed		Exposed		Total
	Non-vaccinated	Vaccinated	Non-vaccinated	Vaccinated	
Sheep sera	419[a]	12[b]	172[c]	100[d]	703
Pig sera	130[a]	54[b]	15[e]	70[f]	269

[a] Field sera originated from Belgium, Denmark, Italy, The Netherlands, United Kingdom.

[b] Experimental sera from United Kingdom and The Netherlands.

[c] Post-outbreak sheep from United Kingdom (no. 100) and Israel (no. 63) known to have been infected according seropositivity to FMDV structural proteins type O; no. 9 experimental sheep from United Kingdom collected 25–28 days post-infection.

[d] No. 78 field sheep from Turkey with unknown infectious status, collected for serosurveillance in affected regions; no. 16 sheep collected 6–8 days post-experimental infection (The Netherlands); No. 6 sheep collected 28 days post-experimental infection (UK).

[e] Sera from 12 pigs collected 21–41 days post-infection, 3 of them twice (UK).

[f] Sera from 55 pigs (UK, The Netherlands) collected between 4–116 days after experimental infection, 5 of them sequentially bled.

samples had been collected in the field or from experimental animals. Samples collected sequentially after infection were available for several of the experimentally infected cattle.

The field samples included 675 sera from 672 naïve animals representing the FMD negative populations of several European countries and 867 sera collected for post-outbreak surveillance in Israel and Zimbabwe. The former were collected from 465 cattle, sampled in four vaccinated herds in Israel between 30 and 80 days after outbreaks of type O [11], and the latter from 402 cattle, collected in six herds with various vaccination statuses in Zimbabwe, from 1 to 5 months after outbreaks of type SAT1 or SAT2 [12].

Sera from experimental cattle ($n = 1037$) included: (i) one sample collected at 3–4 weeks after a single vaccination from each of 425 cattle; vaccination having been with a vaccine manufactured in Europe in the last 15 years and administered as a full or a divided dose; (ii) 62 sera from non-vaccinated cattle exposed to FMDV infection; of which, 21 sera were from 17 cattle that developed a persistent infection (carriers), while 41 sera were from 37 cattle with a negative or an undefined carrier status; (iii) 550 sera from FMDV-vaccinated-and-exposed cattle: of which 225 sera derived from 67 cattle that developed a carrier state, including 17 cattle sampled in South America, between 40 and 60 days after outbreaks of type O or A, except one sample that was collected at 274 days post-outbreak; the remaining 325 sera were derived from 218 cattle with a negative or an undefined carrier status. A serum sample was attributed to the "carrier" category if it had been collected from cattle that had been proven to have developed a persistent infection, defined by positive virus isolation and/or RT-PCR at or beyond 28 days post-exposure.

The interval between vaccination and challenge was usually three or occasionally 4 weeks. Cattle had been exposed to FMDV by inoculation or contact with infected animals or their aerosols. Samples were collected between 7 and 168 days after infection. Table 3 shows the numbers of sera stratified by time after infection.

2.2.2. Sheep sera

A single serum sample was available from each of 703 sheep. The panel of sheep sera (Table 4) comprised 431 sera from non-exposed animals, of which 419 represent the naïve field populations in Belgium ($n = 93$), Denmark ($n = 96$), Italy ($n = 96$), The Netherlands ($n = 19$) and the UK ($n = 115$), while 12 sera originated from non-exposed, experimentally vaccinated animals in UK ($n = 6$) and The Netherlands ($n = 6$). The other 272 sera were from sheep exposed to one of three serotypes of FMDV: O, A, C, either in the field or experimentally. In this last group, 172 sera came from non-vaccinated animals, including post-outbreak sera from a flock in Israel ($n = 63$) [11] and from the UK ($n = 100$). The animals were known to have been infected according to seropositive reactions to serotype O FMDV structural proteins. Sera were also available from nine experimentally infected sheep (UK) sampled 25–28 days post-infection. The remaining 100 sera originated from vaccinated animals; this last group comprising 16 samples collected 6–8 days post-experimental infection in The Netherlands, 6 samples collected 28 days post-experimental infection in the UK and 78 samples from FMD affected regions in Turkey, collected from sheep with an unknown infection status.

2.2.3. Pig sera

The 269 pig sera (Table 4) came from FMDV-naïve pigs or from pigs vaccinated and/or exposed to FMDV-serotype O. Unique sera were available from naïve pigs from the field in Denmark ($n = 40$) and Italy ($n = 40$) or from experimental animals in The Netherlands ($n = 50$). Sera from non-exposed, experimentally vaccinated animals came from the UK ($n = 5$) and The Netherlands ($n = 49$). The 85 sera from pigs exposed to FMDV comprised only experimental samples. Of these, 15 sera were derived from 12 non-vaccinated animals collected in the UK at 21–41 days post-infection (three of them sampled twice), while 70 sera were from 55 vaccinated pigs collected between 4 and 116 days after challenge; 5 of them sequentially bled.

2.3. Databank

Information on each serum sample was stored in a Microsoft Excel database. Each serum was encoded in the databank with a unique number. Besides the results of all six NSP-ELISAs, information was entered concerning the identification of the animal and the serum, the country of origin, whether there was only one sample available from the same animal or sequential samples, the experimental or field origin of the serum, results of previous laboratory tests such as virus isolation, PCR, assays for antibodies to structural proteins, information on infection experiments (route of infection, virus used, clinical signs, whether prior vaccination had been protective), vaccination status and vaccines used, carrier status, the time elapsed between vaccination and/or infection or outbreak and the collection of the blood sample. However, the tests were performed blind without reference to any of this information.

2.4. Diagnostic specificity and sensitivity

A total of 1100 sera from non-infected cattle, 431 sera from non-infected sheep, and 184 sera from non-infected pigs were used to calculate the specificity of the different ELISAs. These included non-vaccinated animals and experimental animals that received a single vaccination (as shown in Tables 2 and 4).

Only sera from experimentally infected animals, representative of different conditions in which vaccinated or non-vaccinated animals may be found after infection with FMDV, were used to calculate diagnostic sensitivity.

For cattle, the analysis was split into four distinct periods corresponding to the following time-intervals: 7–14 days after infection (dpi), followed by the period 15–27 dpi, indica-

tive of very early and early seroconversion, respectively; then the interval 28–100 dpi, corresponding to the appropriate period for serosurveillance after emergency vaccination, and finally a late period, i.e. more than 100 dpi. For those animals that were sampled sequentially after infection, only one sample in each time-category was randomly selected for the analysis. Consequently, in some cases the same animal may be present in two or more periods. Unique samples from sheep and pigs were selected in the same way.

The proportion of positive reactors identified by the tests in each sub-population was calculated with 95% confidence intervals (CI) derived using the method described by Collet [13]. To determine whether the results with different ELISAs were similar, the positive and negative results were analysed using a Fisher-exact test (StatXact®). A significant result ($p < 0.05$) obtained with this test shows that the distribution of positive and negative results for different ELISAs were not similar and indicates that one or more ELISAs perform significantly better or worse than the others.

2.5. Correlation analysis

The conditional dependence between the six tests was expressed in terms of the covariances for sensitivity and specificity (observed value and percent of maximum possible covariance) as described by Gardner et al. [14]. The hypothesis of conditional independence was investigated using a Chi-square (χ^2) test. The analysis was stratified for vaccination status and conducted using all available data for the given combination of two tests, for which the infection status was known. The quantitative results were compared for all combinations of two tests by multivariable linear regression analysis, adjusted for infection status, vaccination status (yes/no) and days post-infection (according to the four time-intervals defined above). The significance of the regression coefficients was assessed by Wald test *p1*-values.

2.6. Evaluation of analytical sensitivity

Two fold dilutions of two positive bovine sera, ranging from neat to 1/128, were prepared in normal negative bovine serum and freeze-dried, with the objective to select International Reference Sera for NSP testing. Serum A had been collected 13–15 days after clinical signs in an animal naturally infected with FMD virus serotype A in an area where vaccination with a trivalent vaccine (O, A, C) had been performed. Serum B had been collected 12 days after intradermolingual inoculation of an animal with FMDV A24 Cruzeiro. The two sets of samples, corresponding to dilution series labelled A1–A8 and B1–B8, respectively, were tested simultaneously with the six NSP-ELISAs on at least two occasions, at a 3 months interval.

2.7. Discrepancy analysis

Results obtained with field sera from Israel and Zimbabwe were assessed by discrepancy analysis and by combined assay performance. Discrepancy analysis [15] was performed by classification of sera with discrepant results in one or more tests in five levels of concordance according to whether positive results were detected in five, four, three, two or one of the six tests. The influence of each test on the distribution of positive results in each class was analysed. The comparative sensitivity was calculated by combined assay performance considering a serum reactive in at least four of the six tests as a true positive.

3. Results

3.1. Comparative diagnostic specificity of the six NSP-ELISA for cattle

Specificities shown by the various ELISAs in non-vaccinated ($n = 675$) and vaccinated ($n = 425$) cattle do not differ significantly, except in the Ceditest ($p = 0.0032$, Fisher-exact test) that showed a higher specificity in vaccinated animals (Table 5). This observation justified the combination of non-vaccinated and vaccinated populations to estimate the specificities of the different assays; after combining the two cattle categories, specificities ranged from 97.2% (CI ⟨96.0%,

Table 5
Diagnostic specificity in non-vaccinated and vaccinated cattle

Vaccination status	No. of cattle	NCPanaftosa screening (%)	IZS-Brescia (%)	Ceditest (%)	Svanovir (%)	Chekit (%)	UBI (%)	*p*-Value Fisher-exact test
First test								
Non-vaccinated	675	97.3	97.3	97.2	98.7	98.2	99.0	0.065
Vaccinated	425	96.9	97.4	99.5	98.1	96.7	97.9	**0.035**
All	1100	97.2	97.4	98.1	98.5	97.6	98.5	0.122
Re-test of positive-reacting sera								
Non-vaccinated	675	98.8	99.9	99.0	99.1	98.8	99.4	0.087
Vaccinated	425	97.4	99.5	99.5	98.8	98.8	98.4	0.067
All	1100	98.3	99.7	99.2	99.0	98.8	99.0	**0.07**

Bold character: significant differences between tests.

Table 6
Comparative detection rates in non-vaccinated and vaccinated cattle exposed to experimental infection

Days after exposure	No. of cattle[a]	NCPanaftosa-screening (%)	IZS-Brescia (%)	Ceditest (%)	Svanovir (%)	Chekit (%)	UBI (%)	*p*-Value Fisher-exact test
Non-vaccinated cattle exposed to infection (no. 54)								
7–14	5	100.0	100.0	100.0	100.0	100.0	100.0	1.000
15–27	27	100.0	100.0	100.0	100.0	100.0	100.0	1.000
28–100	26	100.0	100.0	100.0	96.2	92.3	100.0	0.434
>100	2	100.0	100.0	50.0	50.0	50.0	50.0	0.700
Vaccinated cattle exposed to infection (no. 285)								
All cattle, with or without evidence of infection (no. 285)								
7–14	180–181	48.6	52.2	48.6	40.9	50.0	32.0	**0.001**
15–27	131	60.3	55.7	52.7	49.6	52.7	38.2	**0.012**
28–100	107–108	69.4	64.8	63.6	58.3	50.0	56.1	0.059
>100	47	72.3	63.8	74.5	57.4	38.3	46.8	**0.001**
Cattle with evidence of infection (no. 164)								
7–14	89	56.2	55.1	53.9	47.2	57.3	46.1	0.545
15–27	97	70.1	66.0	61.9	56.7	61.9	47.4	**0.028**
28–100	91–92	75.0	67.4	68.1	57.6	53.3	61.5	**0.029**
>100	47	72.3	63.8	74.5	57.4	38.3	46.8	**0.001**
Only carriers (cattle known to be persistently infected at 28 days or later, no. 67)								
7–14	31	54.8	54.8	54.8	51.6	54.8	38.7	0.781
15–27	36	72.2	66.7	63.9	55.6	58.3	58.3	0.695
28–100	66[b]	93.9	86.4	86.4	71.2	68.2	77.3	**0.001**
>100	37	89.2	78.4	89.2	70.3	48.6	59.5	**0.000**
Cattle with evidence of infection, non-carriers (no. 26)								
7–14	24	8.3	8.3	4.2	8.3	8.3	8.3	1
15–27	23	21.7	8.7	13.0	13.0	8.7	4.3	0.6353
28–100	25–26	26.9	19.2	24.0	23.1	15.4	20.0	0.9367
>100	10	10.0	10.0	20.0	10.0	0.0	0.0	0.8901
Cattle with no evidence of infection (no. 17)								
7–14	15	40.0	46.7	20.0	53.3	33.3	26.7	0.4544
15–27	8	25.0	12.5	12.5	12.5	12.5	0.0	0.9786
28–100	15	33.3	46.7	26.7	66.7	26.7	20.0	0.1067
>100	0	–	–	–	–	–	–	–

Bold character: significant differences between tests.
[a] The same animal may be present once in different time-categories.
[b] Including four cattle with a marginal carrier status and negative for all tests at any time they have been bled, except one sample borderline in the Svanovir test at 49 dpi.

98.1%⟩) to 98.5% (CI ⟨97.6%, 98.8%⟩) at the first screening test. No significant difference between the tests was found for this combined population, as confirmed by the overlapping confidence intervals.

After re-testing the positive-reacting samples (Table 5, second half), the majority were not confirmed as positive and specificity improved in all tests, now ranging from 98.3% (CI ⟨97.3%, 98.9%⟩) to 99.7% (CI ⟨99.2%, 99.9%⟩). The difference between vaccinated and non-vaccinated cattle previously seen in the Ceditest disappeared, but differences between the six ELISAs became significant, as confirmed by the non-overlapping confidence intervals, with IZS-Brescia and Ceditest ELISAs having the highest specificities, 99.7% (CI ⟨99.2%, 99.9%⟩) and 99.2% (CI ⟨98.5%, 99.6%⟩), respectively.

In general, false-positive samples recorded in non-infected cattle exhibited borderline values and a serum reacting as false-positive in one test was in the majority of cases negative in the other five tests (see also specificity covariance under Section 3.3).

3.2. Detection rates of the six NSP-ELISA in FMDV-exposed cattle

The panel of 612 sera described in Table 3 was used to estimate diagnostic sensitivities for the tests in cattle. For the analysis of tests results, the sera were divided into four time intervals, as the strength of serological response depends in part upon the time elapsed since infection. Animals were also categorised according to whether or not they had previously been vaccinated (Table 6).

3.2.1. Non-vaccinated cattle exposed to infection (n = 54)

Most of the tests scored 100% of these sera as positive and there were no statistically significant differences between test

results. The number of cattle examined after 100 dpi ($n = 2$) was insufficient to reach any conclusions for this category.

3.2.2. Vaccinated cattle exposed to infection (n = 285)

The detection rates of the different ELISAs for all of the available vaccinated and challenged cattle, regardless of their infectious status ($n = 285$), are considerably lower than those obtained for the unvaccinated-infected group, and significant differences between the tests were detected.

However, due to the different conditions in which vaccinated cattle may be found after exposure, the following sub-sets of vaccinated-and-challenged cattle were analysed separately: (i) animals in which infection was demonstrated post-challenge (measured by detection of clinical signs, virus recovery, antibody boost against structural proteins), regardless of carrier status ($n = 164$); (ii) "carriers", i.e. cattle that were shown to be persistently infected at or after 28 days post-challenge ($n = 67$); (iii) animals that developed infection after challenge but not a carrier status ($n = 26$); (iv) animals that did not show any evidence of infection after challenge ($n = 17$).

Shortly after exposure (up to 27 dpi), corresponding to the period for seroconversion to NSP, the proportion of seropositive reactors detected by each ELISA was similar for the category including all cattle that had developed infection and for the selected sub-group of carriers. For these categories of animals, an increase in detection rate was observed between 14 and 27 dpi with most of the ELISAs, and for three of them, the OIE Index test NCPanaftosa-screening, the in-house test IZS-Brescia and the commercial kit Ceditest, the proportion of cattle scored seropositive exceeded 60%. After 28 dpi, significant differences in detection rate were evident between ELISAs, including their ability to identify carriers that are considered a high risk for perpetuating infection. In the interval 28–100 dpi, corresponding to the most critical period for post-outbreak surveillance, the carrier detection rate increased considerably for all ELISAs and the superiority of three tests was confirmed: the NCPanaftosa-screening achieved the highest sensitivity of 93.9% (CI ⟨85%, 98%⟩), closely followed by the IZS-Brescia and Ceditest ELISAs with a sensitivity of 86.4% (CI ⟨76%, 94%⟩). After 100 dpi the antibody detection rates did not change significantly for four of the six assays, but some decrease was evident with the Chekit and the UBI ELISAs.

In general, the Chekit ELISA appeared to be one of the more sensitive assays for the detection of early antibodies, but became increasingly less sensitive compared to the other ELISAs at detecting antibodies present after 28 dpi and still more so after 100 dpi.

Sensitivities for carrier detection were potentially reduced by the inclusion in the analysis of four cattle with a marginal carrier status that most likely present a very low risk for virus transmission. These four animals were not scored positive by any of the six ELISAs, except one sample (animal ID 9430 in Table 7) that scored borderline in the Svanovir ELISA at 49 dpi. Experimental details, reported in Table 7, provide evidence that the carrier status of these four cattle is either extremely sporadic, as in the case of animal 1368, or transient, extending not later than 49 dpi in the worst case. Excluding these samples from the analysis, sensitivity values for the carrier animals in the interval 28–100 dpi would increase to 92% with IZS-Brescia and Ceditest ELISAs and reach 100% with the NCPanaftosa-screening.

When only cattle that developed infection, but not a carrier condition are selected ($n = 26$), the detection rates dropped drastically in all tests.

The panel of vaccinated-and-challenged cattle also comprised 17 cattle that did not show evidence of infection after challenge (absence of clinical signs, virus never recovered by virus isolation or RT-PCR, absence of antibody boost). Nevertheless, some sero-reactors to NSP, with concordant results between assays, were found in this group as well, suggesting that NSP testing may discover an infection condition that could be missed by any other testing system.

The results presented in the above analysis were derived from testing all sera once only (i.e. without replication or repetition) with each of the six ELISAs. To allow for the possibility of human errors while assays were being performed, sera that gave discordant results in one or more tests were re-tested. Re-testing these sera usually removed the evident inconsistencies in the results, but did not significantly alter estimates of diagnostic sensitivity.

3.3. Analysis of correlation

The covariance for all combinations of tests for samples taken from infected (sensitivity covariance) and uninfected (specificity covariance) animals is given in Tables 8 and 9, respectively. There is sensitivity covariance for all of the six ELISAs. Relative to the maximum possible covariance, this was lowest, but still highly significant, for Chekit and Svanovir ELISAs in vaccinated animals (67%). In general, higher covariance values were found for non-vaccinated ani-

Table 7
Carriers not detected by NSP tests

Animal ID	Vaccine	Virus challenge	Challenge route	Days post-vaccination challenged	Days post-challenge serum tested	Clinical signs	Detection by virus isolation at dpc	Detection by RT-PCR at dpc
1368	O Manisa	O Manisa	IDL	21	22, 85, 155	No	57, 113	113
9430	A24	A24	IDL	21	21, 49	No	49	28
UV2	O Manisa	O UKG 2001	Contact	21	10, 21, 28, 147	No	4, 7, 10	4, 7, 21, 35, 42
UV14	O Manisa	O UKG 2001	Contact	21	10, 21, 28, 105	No	4, 7, 10, 14, 21, 42	4, 7, 21

Table 8
Sensitivity covariances (and percent of maximum possible covariance) for six FMD NSP-ELISAs by vaccination status[a]

	Chekit	Ceditest	UBI	IZS Brescia	NCPanaftosa-screening	Svanovir
Chekit		0.15 (75%)**	0.161 (74%)**	0.183 (92%)**	0.168 (91%)**	0.16 (67%)**
Ceditest	0.101 (100%)**		0.16 (92%)**	0.189 (77%)**	0.196 (87%)**	0.164 (78%)**
UBI	0.101 (100%)**	0.104 (100%)**		0.168 (97%)**	0.159 (99%)**	0.153 (74%)**
IZS Brescia	0.09 (100%)**	0.093 (100%)**	0.093 (100%)**		0.203 (89%)**	0.176 (84%)**
NCPanaftosa-screening	0.066 (83%)**	0.068 (84%)**	0.068 (84%)**	0.069 (84%)**		0.167 (86%)**
Svanovir	0.099 (88%)**	0.103 (100%)**	0.103 (100%)**	0.091 (100%)**	0.067 (84%)**	

[a] Results for vaccinated and non-vaccinated animals are given above and below the diagonal, respectively.
* Conditional dependence significant at the 5%-level.
** Conditional dependence significant at the 1%-level.

mals. Linear regression analysis confirmed that the quantitative test results were highly correlated for all pairwise comparisons when adjusting for infection status, vaccination status and dpi intervals (results not shown), suggesting that the strong conditional dependence cannot be explained by the stage of infection. In contrast, the specificity covariance ranged from negative, non-significant values (two combinations for vaccinated and eight for non-vaccinated animals) to positive, significant values (eight combinations for vaccinated, three for non-vaccinated animals).

3.4. Comparative analytical sensitivity of the six NSP-ELISAs

Relative analytical sensitivities of the six NSP-ELISAs were compared by determining the detection limit of two bovine positive sera, serially diluted in negative serum. Each dilution was analysed as an individual sample and results were plotted (Fig. 1).

In all ELISAs, the signal recorded with the dilution series of serum B was lower than that produced by the dilution series of serum A, but differences in the detection limit were observed for some of the tests, with no clear correlation between the relevant analytical and diagnostic sensitivities.

With respect to serum A, four ELISAs (Chekit, IZS-Brescia, NCPanaftosa-screening, Ceditest) had a similar limit of detection, all scoring the highest dilution examined (1/128) still positive, although close or coincident with the threshold for detection. The detection limits recorded for Svanovir and UBI tests were two-, and eight-fold lower, respectively.

With regard to serum B, a higher divergence in analytical sensitivity of the six assays was observed: the highest dilution that tested positive was 1/64 for Chekit and IZS-Brescia ELISAs, 1/32 for the NCPanaftosa-screening, 1/16 for the Svanovir test, 1/8 for the Ceditest and 1/2 for the UBI test: thus, a 32-fold difference between the highest and the lowest analytical sensitivity was observed for this serum.

In terms of repeatability, satisfactory results were obtained for all assays, with most fluctuation observed for samples that give rise to high optical densities, which are the strong positive samples in the five indirect ELISAs and weak positive samples in the Ceditest ELISA that is based on competition.

3.5. Comparative performance of the six NSP-ELISAs in post-outbreak surveillance and analysis of discrepancies between assays

Sera from animals in the field may represent the target population for which NSP-ELISAs are intended better than experimentally derived samples. Therefore, the performance of the six tests was compared with 867 bovine sera collected for surveillance after post-vaccination outbreaks involving serotypes O, SAT1 or SAT2. Seroprevalence rates observed in the Israel survey ($n = 465$) varied from 15.7% up to 25.8%. Those from Zimbabwe ($n = 402$) were higher in all tests (ranging from 48.8% up to 67.7%), suggesting a greater degree of virus circulation, consistent with less effective vaccination. In both surveys, the highest detection rates were recorded for the three tests NCPanaftosa-screening, IZS-Brescia and Ceditest ELISAs (Table 10).

The analysis of concordant/discordant results showed that 12.3% of sera from Israel gave concordant positive results in all of the ELISAs and 66.0% were concordance negative. The equivalent figures for the Zimbabwe samples were

Table 9
Specificity covariances (and percent of maximum possible covariance) for six FMD NSP-ELISAs by vaccination status[a]

	Chekit	Ceditest	UBI	IZS Brescia	NCPanaftosa-screening	Svanovir
Chekit		0.005 (100%)**	0.009 (43%)**	0.009 (34%)**	0.004 (12%)*	0.002 (10%)
Ceditest	0.001 (6%)		0.002 (49%)**	0.002 (49%)**	0.000 (−3%)	0.000 (−2%)
UBI	0.001 (13%)*	0.001 (12%)		0.002 (9%)	0.006 (31%)**	0.002 (11%)*
IZS Brescia	0.001 (6%)	0.004 (14%)**	0.000 (−3%)		0.002 (6%)	0.002 (10%)
NCPanaftosa-screening	0.000 (−3%)	−0.001 (−3%)	0.003 (27%)**	−0.001 (−3%)		0.002 (10%)
Svanovir	0.000 (−2%)	0.000 (−3%)	0.000 (−1%)	0.000 (−3%)	0.001 (9%)	

[a] Results for vaccinated and non-vaccinated animals are given above and below the diagonal, respectively.
* Conditional dependence significant at the 5%-level.
** Conditional dependence significant at the 1%-level.

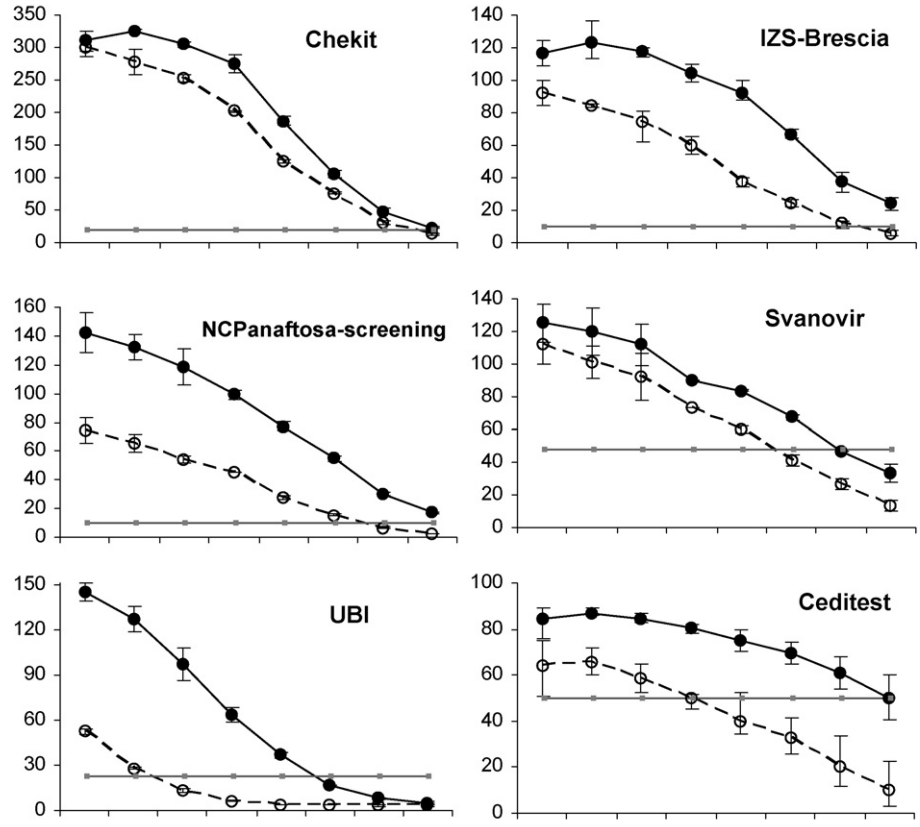

Fig. 1. Dose–response curves of two-fold dilution series of cattle sera A and B. *x*-axis: samples A1–A8 (solid line), samples B1–B8 (dotted line), corresponding to two-fold dilution series from 1/1 to 1/128 of serum A and B, respectively; *y*-axis: expression of results, percentage inhibition for Ceditest ELISA, percentage positivity for all other ELISAs; average of two runs for tests Chekit, Panaftosa, Svanovir, UBI ELISAs; average of three runs for IZS-Brescia and Ceditest ELISAs. Bars: minimum and maximum values recorded in different runs. Grey line: cut-off position, according manufacture instructions.

37.6% and 22.9%, respectively. A total of 22.7% and 39.5% of results were discordant in one or more tests in the two surveys (Table 10).

To explore the origin of these discrepancies, sera were categorized in five levels of concordance. Classes comprised sera giving positive results in five, four, three, two, or only one ELISA out of six. Table 11 shows the relative contribution of each ELISA to the various classes and two tendencies could be observed. The fall in detection rates between the classes representing five tests positive and three tests positive is more abrupt for the Chekit, Svanovir and UBI ELISAs, whereas detection rates of the NCPanaftosa-screening, IZS-Brescia and Ceditest ELISAs maintained a homogeneously high percentage in these classes. This difference in detection rates is particularly evident for the class representing three concordant-positive results. Overall, NCPanaftosa-screening, IZS-Brescia and Ceditest ELISAs yielded a higher contribution of positive results in all classes with two or more concordant-positive results. For sera positive in only one ELISA, there was less difference in detection rates. Several of these reactions may be non-specific, although the possibility of a true positive serum detected only by one test, more sensitive to discover that particular serum condition, test cannot be ruled out. In contrast, the sera giving concordant-positive results in three to five tests, most probably represent truly infected animals. Consequently, the differences in detection rates for these sera most likely represent differences in the sensitivities of the various tests.

In order to define relative sensitivities for the six NSP-ELISAs, and taking into account that the infection status of each animal from which these field samples derived was not

Table 10
Seroprevalence rates (%) and level of agreement for the six NSP-ELISAs in the assessment of cattle sera collected for post-outbreak surveillance

Origin	No. of cattle	NCPanaftosa screening	IZS-Brescia	Ceditest	Svanovir	Chekit	UBI	Level of concordance (%)		
								Positive in six tests	Negative in six tests	Discordant
Israel	465	25.8	25.6	22.4	21.3	20.9	15.7	12.3	66.0	22.7
Zimbabwe	402	67.7	65.2	66.2	50.5	48.8	53.5	37.6	22.9	39.5

Table 11
Detection rates (%) found with the six NSP-ELISAs for the assessment of field cattle sera distributed according levels of concordance

Class	No.	NCPanaftosa screening	IZS-Brescia	Ceditest	Svanovir	Chekit	UBI
Field samples from Israel							
Five tests positive	26	100	92.3	100	88.5	84.6	34.6
Four tests positive	11	100	100	90.9	27.3	63.6	18.2
Three tests positive	10	80.0	80.0	80.0	40.0	10.0	10.0
Two tests positive	12	66.7	58.3	25.0	33.3	8.3	8.3
One test positive	42	23.8	28.6	0	19	21.4	7.1
Field samples from Zimbabwe							
Five tests positive	46	100	100	91.3	78.3	52.2	78.3
Four tests positive	35	97.1	100	88.6	20	40.0	54.3
Three tests positive	20	95.0	95.0	75.0	15.0	15.0	5.0
Two tests positive	20	60.0	35.0	75.0	10.0	5.0	15.0
One test positive	38	26.3	10.5	31.6	10.5	7.9	13.2

Table 12
Relative sensitivities (%) of the six NSP-ELISAs for the assessment of field cattle sera, based on the assumption that sera positive in at least four out of six tests are true-positive

Origin	No.of sera[a]	NCPanaftosa screening	IZS-Brescia	Ceditest	Svanovir	Chekit	UBI
Israel	94	100	97.9	98.9	88.3	91.5	72.3
Zimbabwe	232	99.6	100.0	96.6	83.6	81.5	88.8
Total	326	99.7	99.4	97.2	85.0	84.4	84.0

[a] All field cattle sera that gave positive results in at least four of the six tests.

always known, a serum was considered as true-positive if at least four of the six tests were positive. This assumption is supported by the fact that no positive results in more than two tests were found for naïve populations (data not shown). The sera with three positive tests were not included to minimize the chances of including non-specific reactions. Using this combined assay performance, the NCPanaftosa-screening and IZS-Brescia ELISAs showed the highest relative sensitivities (99.7% and 99.4%, respectively), closely followed by the Ceditest (97.2%). Lower values of 84–85% were shown by the other commercial kits (Table 12) and even these are probably over-estimates since these tests failed to score many sera as positive that were detected by the other three ELISAs. Some sera that are only detected by one to three tests are certainly true positives, as shown by the results from Zimbabwe field animals where 15.1% of the samples scored positive in only one to three tests (data not shown) despite originating from cattle with virus-positive oropharyngeal fluids [12].

3.6. Diagnostic performances of NSP-ELISAs in sheep and pigs

According to the capability of the different NSP-ELISAs to analyse sera from other animal species, four of the six were used to test pigs and sheep in addition to cattle, namely the NCPanaftosa-screening, IZS-Brescia, Ceditest and Chekit ELISAs; the UBI-ELISA was included for testing of pigs. Although the number of experimental sera from sheep and pigs was limited, a preliminary evaluation of the diagnostic performances of these assays with these species was carried out. A total of 431 non-infected sheep and 184 non-infected pigs were analysed to estimate and compare the diagnostic specificities. In sheep (Table 13) specificity was homogeneously high in the IZS-Brescia (99.5 %, CI ⟨98.3%, 99.9%⟩), Chekit and Ceditest (100%, CI ⟨99.2%, 100%⟩) ELISAs, followed by the NCPanaftosa-screening (98,0%, CI ⟨96.3%, 99.2%⟩). In pigs (Table 14) four tests showed high speci-

Table 13
Specificity and detection rates in sheep

NSP-ELISAs	Non-infected (non-vaccinated and vaccinated)		Experimentally infected[a]				
			Non-vaccinated		Vaccinated		
			25–28 dpi		28 dpi		6–8 dpi
	Positive/total	Specificity (%)	Positive/total	Detection rate (%)	Positive/total	Detection rate (%)	Positive/total
NCPanaftosa	8/416	98	9/9	100	3/6	50	4/16
IZS-Brescia	2/428	99.5	9/9	100	4/6	66.6	2/16
Ceditest	0/431	100	9/9	100	4/6	66.6	5/16
Chekit	0/431	100	9/9	100	2/6	33.3	1/16

[a] Undefined infection/carrier status.

Table 14
Specificity and detection rates in pigs

NSP-ELISAs	Non-infected (non-vaccinated and vaccinated)		Experimentally infected[a]				
			Non-Vaccinated		Vaccinated		
			>20 dpi		>20 dpi		≤14 dpi
	Positive/total	Specificity (%)	Positive/total	Detection rate (%)	Positive/total	Detection rate (%)	Positive/total
NCPanaftosa	2/170	98.8	12/12	100	11/16	68.7	3/40
Ceditest	0/152	100	12/12	100	10/18	55.5	5/42
IZS-Brescia	1/179	99.4	12/12	100	8/18	44.4	3/40
Chekit	1/178	99.4	12/12	100	8/18	44.4	2/42
UBI	5/184	97.3	12/12	100	9/18	50.0	3/42

[a] Undefined infection status.

Table 15
Seroprevalence rates in sheep samples from affected areas

NSP-ELISAs	2004 outbreaks (non-vaccinated/positive to SP, Israel)		2001 outbreaks (non-vaccinated/positive to SP, United Kingdom)		Serosurveys in vaccinated areas (Turkey)[a]	
	Positive/total	Seroprevalence (%)	Positive/total	Seroprevalence (%)	Positive/total	Seroprevalence (%)
NCPanaftosa	59/63	93.6	85/100	85	37/78	47.4
IZS-Brescia	59/63	93.6	77/100	77	33/78	42.3
Ceditest	56/63	88.9	83/100	83	36/78	46.1
Chekit	58/63	92	40/100	40	38/78	48.7

[a] Possible bias due to pre-selection of samples on the basis of preliminary testing.

ficities, ranging from 98.8% (CI ⟨95.8%, 99.9%⟩) for the NCPanaftosa-screening to 100% (CI ⟨97.6%, 100%⟩) for the Ceditest, while the specificity of the UBI-ELISA was lower (97.3%, CI ⟨93.8%, 99.1%⟩). It should be noted that these results were obtained with a single test and, as observed with results for cattle, specificities should further improve by re-testing false-positive reactors.

Conclusive data concerning sensitivity cannot be derived for ovine and swine species given the small number of experimental samples; however, the results were in the same range as observed for analogous conditions in cattle. A detection rate of 100% was confirmed with any test later than 20 dpi in both sheep and pigs in the absence of a preceding vaccination. In vaccinated animals (6 sheep, 18 pigs) exposed to experimental infection but with unknown infection status, collected from 20 dpi, considerably lower detection rates were recorded, ranging from 33.3 % (CI ⟨4.3%, 77.7%⟩) to 66.6 % (CI. <22.3%, 95.7%⟩) in sheep and from 44.4 % (CI ⟨21.5%, 69.2%⟩) to 68.7% (CI ⟨41.3%, 89.0%⟩) in pigs according to the different assays. Relatively higher detection rates were obtained with IZS-Brescia and Ceditest ELISAs for sheep and with NCPanaftosa-screening and Ceditest ELISAs for pigs (Tables 13 and 14). There is also evidence of detectable seroconversion earlier than 14 dpi in a minority of vaccinated animals exposed to infection, but this time-period is not optimal for an evaluation of NSP-test performances in relation to post-outbreak serosurveillance.

More sheep sera were available from FMD affected areas, collected either as post-outbreak samples in non-vaccinated regions (63 sera from a unique flock in Israel and 100 sera from the 2001 epidemic in United Kingdom, all of them positive for antibodies to FMDV type O structural proteins), or for surveillance in vaccinated areas of Turkey (78 samples). These sera enabled us to assess under field conditions, the diagnostic performances of the four NSP-ELISAs suited for testing sheep (Table 15). The four assays scored a high and homogeneous NSP-seroprevalence in the non-vaccinated flock sampled in Israel, indicative of a uniform and recent infection status of the animals within the herd. In contrast, more variation was observed in the seroprevalence rates detected by the different tests in 100 sheep from the UK epidemic, with higher detection rates shown by the NCPanaftosa-screening and Ceditest ELISAs. This may reflect a longer interval between infection and sampling in the UK. Consistent with results from the experimentally derived sera, detection rates found in vaccinated areas in Turkey are lower than those recorded in non-vaccinated regions; however, they are similar for all assays and provide evidence of virus circulation.

4. Discussion

The objective of this study was the evaluation of four commercial ELISAs and one in-house test developed in European laboratories. These tests were compared for their ability to detect animals that had been or continued to be infected with FMDV, primarily within a vaccinated population and the evaluation was made with reference to the OIE Index method (NCPanaftosa-screening). All six ELISAs are screening tests and need a confirmatory system, consisting of either a confirmatory assay, or a follow-up of epidemiological units showing results positive at the screening test, or a testing system with known performance as suggested by Paton et

al. [16]. The performance of the ELISAs will be of interest to international organisations, like the EC, FAO and OIE, as well as to National Veterinary Authorities, all of whom are involved in the design of scientifically based serosurveillance sampling strategies.

The approach adopted, i.e. collection of sera from several sources and testing in parallel by each of the six ELISAs in a single laboratory during an open workshop, gives transparent results and reduces many potential sources of variability.

The specificities estimated reflect the negative population of several European countries from which the naive sera were originated. The great majority of false-positive reactions gave borderline values. The approach to re-test samples that gave false-positive results led to improved specificities for all ELISAs. All tests are subject to some lack of repeatability and this is why re-testing positives is a widespread practice for all forms of serosurveillance. In the present study, the operating conditions of the first screening test, that involved testing of thousands of samples with different ELISAs without replicates, might justify the improved specificity after re-testing.

In cattle, the specificity of the OIE Index test (98.1%) was lower than those of the other five ELISAs (98.8–99.7%); however, the cut-off of the NCPanaftosa-screening was selected to optimise sensitivity, given the availability of a very specific and equally sensitive confirmatory test, consisting of an immunoblotting assay [17]. In this trial, the highest specificities were provided by the IZS-Brescia and Ceditest ELISAs (99.7% and 99.2%, respectively). For sheep and pigs, specificities approached 100%, even at the first screening.

An analysis of the conditional dependence was conducted using results from cattle, in order to investigate and quantify the specificity covariances. This analysis suggests that false-positive results are in the majority of cases not correlated; the Svanovir ELISA appears to be the most independent among the six tests as far as false-positive reactors is concerned.

Confirming results of previous reports [2,4,8–10,18–21], the specificity of all tests is not affected by a single vaccination with European vaccines. This condition is comparable with an emergency vaccination in countries free of FMD, so that the specificities estimated may be the basis to calculate the proportion of false-positive reactions that can be expected in a serosurvey to demonstrate post-outbreak freedom from infection using these tests [16].

The evaluation of sensitivity is much more complex, since it must consider the several conditions, relevant to both the immunological and infectivity status, that may be found in vaccinated or non-vaccinated animals after infection with FMDV. In non-vaccinated animals exposed to infection the sensitivity of all ELISAs for all of the three animal species evaluated was very high, approaching 100%. Therefore, NSP-ELISAs may be used reliably when emergency vaccination is not applied. In vaccinated animals exposed to infection, the performances of the different ELISAs were not as homogeneous as for unvaccinated ones and detection rates varied according to the time after infection. In general, results indicate that seroconversion against NSP occurs in vaccinated animals more slowly and in a lower proportion than in unvaccinated ones, which is an expected consequence of the limited viral replication in vaccinated animals. Furthermore, as not all vaccinated animals exposed to infection become infected, a reduction of the prevalence of anti-NSP antibodies in the group of animals that includes such condition is obvious. Nevertheless, detection rates of more than 60% were obtained with the three more sensitive tests (NCPanaftosa-screening, Ceditest and IZS-Brescia ELISAs) in experimentally vaccinated-and-challenged cattle, analysed from 28 dpi regardless of their infectivity status. Similar values were also obtained for sheep and pigs, even if the number of experimental samples available from these species was small. Although obtained under experimental conditions, these values may reflect the field prevalence of seropositive animals detectable in a vaccinated population in the case that each vaccinated animal is exposed to FMD virus. The seroprevalence rates detected with the six tests in the Zimbabwe post-outbreak survey are similar to those found in experimental conditions; however, seroprevalence values recorded in Israel were considerably lower (around 25%) and suggestive of lower virus exposure, replication and/or circulation.

From an epidemiological point of view, the category of cattle for which evidence of infection after challenge was available is more interesting. A clear difference is evident between detection of cattle that developed a persistent infection (carriers) and cattle that recovered from infection. In carrier cattle, the detection rates increased gradually after challenge, reaching the highest rates during the time interval 28–100 dpi. In contrast, the proportion of seropositives in cattle that recovered from infection is dramatically lower. This indicates that virus persisting in the oesophago-pharyngeal region induces an effective and sustained immune response to NSP, whilst in animals that recover shortly after infection NSP antibodies are elicited weakly and disappear rapidly. In a serosurvey to demonstrate freedom from infection in regions where a vaccinate-to-live strategy has been applied, cattle recovered from infection, even if not detected by the serological system used, do not represent a risk for spread of FMDV.

In general, the most sensitive test is the NCPanaftosa-screening, that identified 93.9% of carriers at 28–100 dpi; IZS-Brescia and Ceditest ELISAs are equivalent to one another and perform similarly to the Index test, with detection rates of 86.4%. Sensitivities estimated for carriers would have further improved if four cattle with a very transient or sporadic virus recovery are excluded from this evaluation.

Samples were defined as being from carriers if virus had been detected in such animals at or beyond 28 days post-challenge. However, grouping of samples collected between 28 and 100 dpi could underestimate test sensitivities by including sera from transient carriers that had eliminated virus by the time of blood sampling. This did not appear to have occurred, since 5 of the 50 samples collected at 28–100 days after experimental challenge from vaccinated carrier cattle came from cattle where no virus could be detected at or beyond the time of sample collection (data not shown), but all

of these samples were scored as positive in the Index test. Four of the six tests maintained high detection rates in cattle classified as carriers beyond 100 dpi, while the Chekit and UBI ELISAs showed a more evident drop. This different trend may simply reflect test sensitivity, or a diversity in the spectrum of antibodies detectable by the various tests. However, given that a considerable proportion of cattle categorised as carriers were no longer detectably infected when sampled after 100 dpi, the detection rates calculated for this latest period do not properly represent the sensitivity for detecting carriers, but rather reflect the duration of antibodies in cattle that recovered from a persistent infection.

A remarkable finding in favour of NSP-serology is the detection of a proportion of vaccinated-challenged cattle that were scored as positive by all or the majority of ELISAs, in spite of lacking any other evidence of infection (Table 6). This finding suggests that circumstances exist in which NSP-serology is, even on individual animals, more sensitive than virus detection in sequential samples of oropharyngeal fluids, and therefore it may help to detect infection that would be missed by any other testing system.

The three assays with higher diagnostic sensitivity on experimental sera also showed higher detection rates and concordance when applied to field samples. Indeed, NCPanaftosa-screening, IZS-Brescia and Ceditest ELISAs contributed higher numbers of positive-concordant results in the two post-outbreak surveys conducted in Israel and Zimbabwe. Although the results from field samples do not allow one to estimate absolute sensitivity values, they provide estimates of NSP-antibody prevalence in different field situations and a further possibility to compare tests. Furthermore, outbreaks investigated in Zimbabwe were due to SAT1 and SAT2 serotypes, enabling extension of validation of NSP-tests to these serotypes.

The analysis of conditional dependence between the six tests indicates that both the quantitative and the binary (after application of the cut-off value) test results for vaccinated-and-infected and for non-vaccinated-and-infected cattle were highly correlated among all six ELISAs. A significant positive sensitivity covariance indicates that false-negative test results are correlated to some extent. This is a practically relevant finding and provides the basis for correct specifications of the overall sensitivity when combining two (or more) diagnostic tests into a testing strategy [14,16]. It should be noted that the direct estimation of the sensitivity (and specificity) covariance requires the parallel testing of the same serum panels with all involved diagnostic tests and the knowledge of the true infection status for each animal. The design of the present study fulfilled both these requirements.

Evaluation of analytical sensitivity is a normal part of the validation process for diagnostic assays, and was therefore included in the comparative evaluation of the six NSP-ELISAs. Differences were observed between the ELISAs but these did not always correlate with diagnostic sensitivities. Analytical sensitivity is obviously related to the absolute antibody concentration, but may also reflect a variable sensitivity of the assays to reveal antibodies that are distinctive for individual animals. The analytical sensitivity of assays is therefore strongly conditioned by the sera used for this evaluation and these must be selected with care and in sufficient number to represent the variability that can be expected. This could explain the higher divergence observed between analytical sensitivities of the six assays when evaluated with serum B compared to serum A, and the inconsistent correlation with the relevant diagnostic sensitivities. In fact, the Chekit ELISA showed the highest analytical sensitivity, together with IZS-Brescia and NCPanaftosa ELISAs, in spite of a lower diagnostic sensitivity, while the Ceditest ELISA showed a low analytical sensitivity for one of the two sera but a high diagnostic sensitivity. A larger panel of reference sera is therefore needed to evaluate batch-to-batch consistency of these ELISAs and to establish the sensitivity of new tests.

The number and heterogeneity of cattle sera were sufficient to enable a comprehensive comparison of the diagnostic performances of the six ELISAs for use with this species leading to the following principal conclusions:

1. Test results for sera from infected cattle are highly correlated among all the six ELISAs, however the extensive comparison enabled us to graduate the sensitivities of tests available in Europe and compare them to the sensitivity of the OIE index test.
2. The commercial Ceditest kit and the in-house IZS-Brescia test perform comparably to the Index NCPanaftosa-screening ELISA and the highest sensitivities for both experimental and field sera were observed with these three tests.
3. In infected cattle the production of antibodies to NSP is correlated to the extent of viral replication, so that animals that are not protected by vaccination or are carriers are more likely to be detected. In fact, sensitivity reaches 100% with any ELISA for testing non-vaccinated, infected cattle and approaches or exceeds 90% in vaccinated, carrier cattle with the better tests.
4. NSP-tests are currently the most sensitive tool to detect present or past infection with FMDV in vaccinated cattle, after sampling at a single time-point.

More sera from sheep and pigs have to be collated and tested before firm conclusions can be drawn on the specificity and sensitivity of NSP-ELISAs for these species. However, preliminary data based on few experimental and field sera analysed in this study indicate performances similar to those observed for cattle.

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